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CYTOCHROME OXIDASE I-BASED PHYLOGENETIC RELATIONSHIPS AMONG THE POMATIOPSIDAE, HYDROBIIDAE, RISSOIDAE AND TRUNCATELLIDAE (GASTROPODA: CAENOGASTROPODA: RISSOACEA)

George M. Davis[1], Thomas Wilke[1], Christina Spolsky[1], Chi-Ping Qiu[2], Dong-Chuan Qiu[3], Ming-Yi Xia[2], Yi Zhang[2], & Gary Rosenberg[1]

ABSTRACT

The Gondwanian-derived Asian pomatiopsid radiation is taxonomically complex, diversity-rich, and widely deployed geographically. This Asian branch of the family has coevolved with such human trematode parasites as *Schistosoma* and *Paragonimus;* it is ideally suitable for studying patterns and processes of evolution over 100 million years. Cytochrome *c* oxidase subunit I gene sequences are used here to elucidate taxonomic relationships from the subspecies to familial level. In Chinese literature, pomatiopsid taxa have been classified in the Hydrobiidae; what are the genetic relationships between *Hydrobia* and allied taxa classified as pomatiopsid?

Sixteen sequences, ranging in length from 578 to 645 nucleotides, are aligned from 11 species of nine genera assigned to seven families, four of which are rissoacean. Five different phylogenetic analyses are concordant: (1) the pomatiopsid taxa are in one distinct clade, the other rissoaceans form a second clade; (2) truncatellids are more closely allied to the hydrobiids than to the pomatiopsids; (3) the rissoid *Setia* is part of the truncatellid-hydrobiid clade; (4) two subspecies of *Oncomelania* are clearly divergent; (5) triculine taxa appear divergent from pomatiopsine taxa. However, the *Tricula* sp. node is weakly supported.

Individuals of a population differ by an average of 0.005 ± 0.004 nucleotide differences/site; the subspecies of *Oncomelania* differ by 0.148 ± 0.004; the two species of *Hydrobia* differ by 0.162 (range of 0.161 - 0.163); the triculine genera *Tricula* and *Gammatricula* differ by 0.132 (range of 0.130 - 0.133); the pomatiopsid subfamilies Pomatiopsinae and Triculinae differ by 0.179 ± 0.020; the families Hydrobiidae and Pomatiopsidae differ by 0.267 ± 0.016. Non-rissoacean and rissoacean taxa differ by 0.274 ± 0.023.

Key words: systematics, cytochrome *c* oxidase subunit I, COI, gene sequences, phylogeny, Rissoacea, Pomatiopsidae, Hydrobiidae, Truncatellidae, Rissoidae, China, Jamaica, Bulgaria, Denmark.

INTRODUCTION

This study is one of a series (reviewed in Davis, 1992) aimed at understanding the origin and evolution of the freshwater snail family Pomatiopsidae in Asia. There are compelling reasons to pursue such studies: (1) The family is ideally suited for in-depth studies of biogeography and evolution. The family is of Gondwanian origin with genera found in South Africa, South America, northern India, and Australia. Davis (1979) hypothesized an introduction of early pomatiopsids from the northeastern Indian Plate into northern Burma and western China with subsequent distribution throughout southern China, Japan, and the Philippines reaching North America via Bering Strait. (2) Pomatiopsids are ideal for studying patterns and processes of evolution over the past 100 million years. The family is taxon rich and widely deployed geographically. A series of anatomical studies on pomatiopsid taxa have yielded a rich database of characters and character-states enabling the establishment of testable phylogenetic hypotheses of the evolution of the family. Each phylogeny is tested with the addition of data from a newer study. The latest phylogeny (Davis, 1992) has not falsified the previous phylogenies. These phylogenies are mapped on area cladograms testing and reinforcing the biogeographic hypothesis. (3) A diverse array of Asian pomatiopsids are important intermediate hosts of human trematode para-

[1]Academy of Natural Sciences of Philadelphia, 1900 Benjamin Franklin Parkway, Philadelphia, Pennsylvania 19103, U.S.A.; davis@say.acnatsci.org.
[2]Chinese National Center for Systematic Medical Malacology, Chinese Academy of Preventive Medicine, 207 Rui Jin Er Liu, Shanghai 200025, People's Republic of China
[3]Sichuan Institute of Parasitic Diseases, Chengdu 610041 Sichuan, People's Republic of China

sites. These studies are essential to study coevolution of the blood parasite *Schistosoma* with prosobranch snails. Three genera of the Pomatiopsidae — *Oncomelania, Neotricula* and *Robertsiella* — are involved in the transmission of the human blood fluke *Schistosoma* in China, the lower Mekong River, and Malaysia. The schistosome occurring in China is also distributed in Japan, Taiwan, the Philippines and Sulawesi. The genus *Schistosoma* is, as are the Pomatiopsidae, of Gondwanian origin; one branch of the *Schistosoma* clade has coevolved with Asian Pomatiopsidae, with genera distributed in two subfamilies, the Pomatiopsinae and Triculinae (reviewed in Davis, 1980, 1992). (4) Molecular data are essential to test the phylogenetic results based on anatomy. Allozyme data have, to the limited degree they have been applied, reinforced the phylogenetic hypotheses on the relationships of key genera in the aforementioned subfamilies (Davis et al., 1994); that is, the phylogenies based on allozymes and anatomical data are congruent. They have been useful to clarify subspecific status of populations of *Oncomelania hupensis* in China (Davis et al., 1995). But with the need to include numerous taxa for phylogenetic analysis, the ability to use allozyme data effectively decreases. We have found that mitochondrial gene sequences are ideally suited for testing phylogenetic relationships based on anatomical data. We have established that cytochrome *b* sequences are useful to determine patterns of divergence at the population and subspecies level within the genus *Oncomelania* (Spolsky et al., 1996).

There are a number of questions that this study was designed to answer: (1) Are the Hydrobiidae and Pomatiopsidae truly divergent separate families? From Davis (1979) onward we have argued that, on the basis of anatomy and patterns of development, the Hydrobiidae and Pomatiopsidae are highly divergent. This is an important question because up to the present, Chinese workers and others around the world have insisted on including the Pomatiopsidae within the Hydrobiidae and thus believe that some hydrobiids transmit schistosomes (Malek & Little, 1971; Brandt, 1974; Liu et al., 1974; Liu, 1979; Brown, 1980 (revised to use Pomatiopsidae in 1994); Kang, 1984, 1986; Malek, 1985). Davis (1979, 1980, 1992) has shown that the Hydrobiidae are not found in India, China or southeast Asia; further, no hydrobiid transmits *Schistosoma*.

Understanding the patterns of divergence of these two families is important to understanding the origin and coevolution of rissoacean snails with *Schistosoma*. One purpose of this paper is to present additional evidence that the two families are distinct and divergent.

(2) On the basis of anatomical data, there are two distinct subfamilies of the Pomatiopsidae; the Pomatiopsinae and Triculinae. Allozyme data reinforced the confamilial status of the two generic groupings but did not unequivocally serve to demonstrate two distinct subfamilies as anatomical data did (Davis et al., 1994). Would the cytochrome *c* oxidase subunit I (COI) gene sequences serve to clearly demonstrate family and subfamily-level generic groupings?

(3) Would the Truncatellidae (represented here by only one species) be more closely related to the Hydrobiidae or to the Pomatiopsidae? Davis (1979) and Ponder (1988) considered the Truncatellidae to be closely related to the Pomatiopsidae on the basis of anatomical data. Analyses of 28S rRNA sequences involving one species of Hydrobiidae, nine species of two genera of Truncatellidae, and one species of Pomatiopsidae (Rosenberg et al., 1997) placed the Hydrobiidae as an outgroup to a cluster consisting of two branches: one solitary branch included some of the truncatellid taxa; the second branch subdivided into two groups, one including the remaining truncatellid taxa, the other the pomatiopsid species. What would the COI sequence data tell us?

(4) Would COI data support the conclusion based on allozyme data (Davis et al., 1995) that *Oncomelania hupensis hupensis* and *O. hupensis robertsoni* are distinct subspecies?

METHODS

Taxa Studied

Pomatiopsidae: Triculinae: *Gammatricula chinensis* Davis, Liu & Chen, 1990; *Tricula* sp.

Pomatiopsidae: Pomatiopsinae: *Oncomelania hupensis hupensis* (Gredler, 1881); *Oncomelania hupensis robertsoni* Bartsch, 1946.

Hydrobiidae: Hydrobiinae: *Hydrobia* cf. *pontieuxini* Radoman, 1973; *Hydrobia neglecta* Muus, 1963.

Rissoidae: *Setia turriculata* Monterosato, 1884.

Truncatellidae: *Truncatella pulchella* (Pfeiffer, 1839).

TABLE 1. Localities and collecting information for the specimens studied.

Taxa; Preparation #	Localities	Catalog#
Pomatiopsidae:Triculinae		
Gammatricula chinensis 414/415/416	China, Zhejiang Province, Kaiwa Co, Tong Cun Town, Bai Keng Village; 118°15′47″E, 29°00′05″N	ANSP 400351 ZAMIP MO136
Tricula sp. 453/454	China, Sichuan Province, Dayi County; Tian Gong Mia Township; Huang Ba Village; 117°23′16″E, 30°35′26″N	ANSP 400352
Pomatiopsidae: Pomatiopsinae		
Oncomelania hupensis hupensis 93/96	China, Hubei Province, Han Yang County; 114°01′01″E, 30°34′08″N	ANSP 375731
Oncomelania hupensis robertsoni 45/48	China, Yunnan Province; Dali City, Da Jin Ping, Zi Ran Village; 100°12′04″E, 25°27′06″N	CIPD 0349
Hydrobiidae		
Hydrobia cf. *pontieuxini* 346/347/351	Bulgaria, 1 km W of Nessebar; 27°71′73″E, 42°65′99″N	ANSP 400353
Hydrobia neglecta 435/436/439	Denmark, Funen Island, Odense Fjord; 10°32′E, 55°30′N	ANSP 400354
Rissoidae		
Setia turriculata 474/476/477	Bulgaria, 1 km W of Nessebar; 27°71′73″E, 42°65′99″N	ANSP 400355
Truncatellidae		
Truncatella pulchella 479–480	Jamaica, W of Falmouth; 77°39′46″W, 18°29′46″N	ANSP 400356

Four molluscan taxa were used as outgroups: (1) the polyplacophoran *Katharina tunicata* (Wood, 1815), the sequence for which was obtained from GenBank (accession number U09810); (2) the cerithiacean *Cerithium atratum* (Born, 1778) (Harasewych et al., 1997); (3) the muricacean *Stramonita haemastoma* (Linnaeus, 1767) (GenBank accession number U86330, under the name *Thais*); and (4) the rissoacean *Setia turriculata* studied in this paper.

Locality data are given in Table 1. *Oncomelania hupensis hupensis, O. hupensis robertsoni* and *T. pulchella* were brought to the USA alive, *G. chinensis* and *Tricula* sp. were preserved in 100% methanol, *H.* cf. *pontieuxini* and *H. neglecta* were preserved in 70% ethanol, and *S. turriculata* was frozen. Immediately prior to isolation of DNA, the living specimens of *Oncomelania* and *Truncatella* were quick-frozen at -80°C.

DNA Preparation

The methods used for preparing DNA from individual snails were described by Spolsky et al. (1996), with the following modifications. Alcohol-preserved specimens of *Gammatricula*, *Tricula* and *Hydrobia* were soaked 5 min each in two changes of 300 µl of ice-cold exchange buffer before being placed in lysis buffer. Ethanol precipitation and washing were repeated and the final DNA pellet redissolved in 25 µl of water.

The quality of DNA was determined by electrophoresis through a 1% agarose gel in TBE. DNA concentration was determined using a Hoefer TKO100 fluorometer.

DNA Amplification

PCR was used to amplify a fragment of the mitochondrial COI gene using the primer pair COF14 (forward: 5′ GGTCAACAAATCATAAAGATATTGG 3′) and COR722 (reverse: 5′ TAAACTTCAGGGTGACCAAAAAAYCA 3′). COF14 is identical to primer LCO1490 as described by Folmer et al. (1994), while COR722 is a modification of Folmer et al. (1994) primer HCO2198.

Each PCR reaction mixture, in a total volume of 50 µl, contained 20-100 ng of genomic DNA, 2.5 units of cloned Pfu DNA polymerase (Stratagene), 200 µM of each dNTP, 20-40 pmol of each primer, 20 µg of BSA, and 5 µl of 10X Pfu reaction buffer. PCR amplifications

were performed using an M-J Research PTC-100 thermal controller with the following cycling conditions: initial 1 min 30 sec at 95°C, followed by 40 cycles of 1 min at 95°, 1 min 20 sec at 47°C, and 1 min 10 sec incremented by 1 sec per cycle at 73°C. After a final 5 min at 74°C, reactions were held at 4°C.

Amplified DNA products were separated by electrophoresis through a 1% low melting point agarose gel in TAE buffer. The band corresponding to a fragment of the correct size was cut out, and the DNA purified using either Microcon-100 microconcentrators (Amicon) after digesting the agarose with agarase or directly with Wizard PCR preps (Promega). DNA concentration was determined on a Hoefer TKO100 fluorometer.

Sequencing

Sequences of the COI fragment were determined by manual cycle sequencing, using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham) according to their protocol.

Each reaction mixture contained 40-60 ng of purified PCR product, 4 units of Thermo Sequenase DNA polymerase, 2 μl of reaction buffer, 4 pmol of either COF14 or COR722 primer in a total reaction volume of 20 μl. One fourth of the reaction mixture was aliquoted to each of four dideoxynucleotide-specific termination mixes containing the four dNTPs at 7.5 μM each plus, for each termination, 0.15 pmol of one of the four ^{33}P-labelled ddNTPs at a specific activity of 1500 C_i/mmole. Cycling conditions consisted of 30 cycles of 60 sec at 95°C, 60 sec at 51°C and 75 sec at 72°C for the forward primer and 30 cycles of 60 sec at 95°C, 60 sec at 56°C and 75 sec at 72°C for the reverse primer. Four μl of stop solution were added after cycling and samples were stored frozen until ready to use.

Before loading, the samples were heated 5 minutes at 75°C and 3 μl of each loaded immediately on the gel. Reaction products for each primer were run on three separate gels: 2.5 h on an 8% Long Ranger gel (FMC), 4 h on a 6% gel, and 7.5 h on a 5% gel at a constant power of 37.5 Watts, providing complete sequences for both strands.

Data Analyses

COI sequences for each individual were assembled and edited using ESEE 3.0s (Cabot & Beckenbach, 1989). ESEE was also used to align our sequences with sequences obtained from GenBank and the literature.

A distance matrix was computed using DNADIST of PHYLIP version 3.57 (Felsenstein, 1989, 1993). Maximum likelihood trees were generated using DNAML (PHYLIP 3.57). Twenty repetitions, with randomized input order and optimization by global branch rearrangement, were run for each analysis. Bootstrap estimates (1,000 replicates) were made using program SEQBOOT, in conjunction with DNAML and CONSENSE. Parsimony analyses were done using Hennig86 (Farris, 1988) and PAUP 3.0s with branch and bound searching (Swofford, 1993). In analyses using PAUP, terminal nucleotides not present in all sequences were trimmed from the analyses; using DNAML, all 645 nucleotide positions were included.

We performed five different phylogenetic analyses to satisfy different conditions and compare among methods: (1) DNAML with the polyplacophoran *Katharina* and the rissoid *Setia* as outgroups; (2) As the polyplacophoran was not much more distant from the ingroup taxa than was *Setia*, we used *Setia* as the outgroup in a maximum likelihood analysis; (3) Also with *Setia* as the outgroup, we ran Hennig86 (256 variable sites; 220 informative sites); (4) We subsequently added the two Caenogastropoda; these two taxa are better outgroups (i.e., within Gastropoda but outside Rissooidea) than either *Katharina* or *Setia*. To our knowledge, there are no data available for other rissoacean taxa that might serve as outgroups. For this set of taxa, we computed a maximum likelihood tree as follows: first, we empirically determined the optimal transition/transversion ratio (ratio which minimizes the likelihood measure; we then used the optimal ratio, 1.3 in this case, in an exhaustive DNAML analysis (20 iterations, global branch swapping); (5) we also computed parsimony trees for the same set of taxa, using a 1:1 transition: transversion ratio.

RESULTS

Sequence alignments for 16 sequences, ranging in length from 578 to 645 nucleotides, are shown in Table 2. Individuals with identical sequences (414, 415; 474, 476, 477; 346, 347; 435, 436, 439; and 453, 454) were combined for alignment and subsequent analyses. A single nucleotide at position 57 is missing from the *Cerithium* sequence; we pu-

TABLE 2. Alignment of sequences of a 645 nucleotide fragment of the cytochrome *c* oxidase I gene.

Katharina tunicata	ATC	TTG	TTT	GGT	ATT	TGA	GCT	GGG	TTA	GTA	GGG	ACT	GCT	TTA	AGA	TTA	CTA	ATT	CGT	GCA	60
Stramonita haemastoma	---	---	---	---	---	---	---	---	C.G	...	..A	..G	...	...	..T	C.T	..C	...	..G	..T	
Cerithium atratum	---	---	---	---	---	---	---	--T	.GG	A.T	..A	...	...	C.C	..T	C..	T..	...	..N	..C	
G. chinensis 414/415	..T	..A	...	...	..A	...	...	..C	...	..T	..A	...	...	C.T	...	...	T..	...	..A	..T	
G. chinensis 416	..T	..A	...	...	..A	...	...	..C	...	..T	..A	...	...	C.T	...	...	T..	...	..A	..T	
Tricula sp. 453/454	..T	..A	...	..G	..A	...	..A	..C	...	..T	..T	...	...	C.T	...	...	T..	...	..A	..T	
O. hupensis hupensis 93	..T	..A	..C	...	..A	..G	..A	..A	C.T	..T	..C	...	..C	C.T	...	...	T..	...	...	...	
O. hupensis hupensis 96	..T	..A	..C	...	..A	..G	..A	..A	C.T	..T	..C	...	..C	C.T	...	...	T..	...	...	...	
O. hupensis robertsoni 45	..T	..A	...	...	..G	..G	..A	...	...	..T	..T	...	...	C.T	...	...	T.G	...	...	...	
O.hupensis robertsoni 48	..T	..A	...	...	..G	..G	..A	...	...	..T	..T	...	...	C.T	...	...	T.G	...	...	...	
H. cf. *pontieuxini* 346/347	..T	..A	...	...	..G	...	T..	..C	C.G	..C	..T	..A	..A	C..	..T	...	...	...	...	..T	
H. cf. *pontieuxini* 351	..T	..A	...	...	..A	...	T..	..C	..G	..C	..T	..A	..A	C..	..T	...	...	...	...	..T	
H neglecta 435/436/439	..T	..A	...	...	..G	..G	T..	..A	...	...	..T	..A	..A	C..	..T	..G	T..	...	...	..T	
S. turriculata 474/476/477	..T	..A	...	...	..A	...	T..	...	C..	..T	..A	..A	...	...	...	...	..T	...	...	..T	
T. pulchella 479	..T	...	...	..A	..G	..G	T..	..T	C.T	..T	..A	...	..A	...	..T	...	T..	...	..A	..T	
T. pulchella 480	..T	...	...	..A	..G	..G	T..	..T	C.T	..T	..A	...	..A	...	..T	...	T..	...	..A	..T	

GAG	CTA	GGT	CAA	CCA	GGG	GCT	TTA	TTG	GGG	GAT	GAC	CAA	CTG	TAT	AAT	GTT	ATT	GTC	ACA	GCT	CAT	GCT	TTT	GTT	135
...	..C	..G	...	..C	...	...	...	C.T	..A	...	..T	...	T.A	...	...	..A	...	..G	..G	..A	...	...	...	..A	
..A	T..	..A	..G	..T	...	..C	C.T	C.T	..T	...	..T	..G	..A	...	...	..A	...	..A	..G	..A	...	..C	..C	..A	
...	..T	...	...	..T	..A	..A	...	..A	..T	...	..T	...	..T	...	...	...	...	..T	...	..A	...	..A	...	..A	
...	..T	...	...	..T	...	..A	...	..A	..T	...	..T	...	..T	...	...	...	...	..T	...	..A	...	..A	...	..A	
...	T..	..G	...	...	..A	..G	C..	..A	..T	...	..T	...	..T	...	...	..A	...	..T	...	..G	...	..A	...	..A	
...	T..	..A	..G	..T	...	...	..G	C.T	...	...	..T	..G	..C	...	..C	..A	...	..T	...	..A	...	..A	...	..G	
...	T..	..A	..G	..T	...	...	..G	C.T	...	...	..T	..G	..C	...	..C	..A	...	..T	...	..A	...	..A	...	..G	
...	T.G	..G	..G	...	...	...	...	C.T	..A	...	..T	...	..T	...	...	..A	...	..T	...	..A	...	..A	...	..A	
...	T.G	..G	..G	...	...	...	...	C.T	..A	...	..T	...	..T	...	...	..A	...	..T	...	..A	...	..A	...	..A	
..A	T..	..G	..G	...	..T	..A	C.T	..A	...	...	..T	..G	..C	...	..C	..G	...	..A	..T	...	...	...	...	...	
..A	T..	..G	..G	...	..T	..A	C.T	..A	...	...	..T	..G	..C	...	..C	..G	...	..A	..T	...	...	...	...	...	
..A	...	...	..G	..T	..T	..G	C.T	...	..T	...	..T	..G	..T	...	..C	..A	...	..T	..T	...	...	..C	...	...	
..A	...	...	...	..T	..A	...	...	..A	..T	...	...	..G	..T	...	..C	..A	...	..A	..T	...	...	...	...	A..	
..A	T..	..C	...	..G	..T	...	C.C	..A	..T	..C	..T	...	..T	...	...	..A	...	...	..G	...	..C	...	...	..A	
..A	T..	..C	...	..G	..T	...	C.C	..A	..T	..C	..T	...	..T	...	...	..A	...	...	..G	...	..C	...	...	..A	

ATA	ATT	TTT	TTT	TTG	GTT	ATG	CCT	ATA	ATA	ATT	GGG	GGG	TTT	GGT	AAC	TGG	CTA	GTG	CCT	TTA	ATG	TTA	GGG	GTG	210
...	..C	..C	...	..A	...	..A	..C	..G	..G	...	..A	...	...	..C	..T	..A	..G	..T	..C	C.G	..A	...	..T	.CA	
...	...	..C	...	..A	..A	...	..A	...	...	...	..T	..A	...	..C	..T	..A	T.G	..T	..C	C.T	...	..G	..A	.CT	
...	...	...	...	..A	...	...	...	...	..G	...	..T	..T	...	..A	...	..A	..G	A.T	..A	...	...	...	...	.CT	
...	...	...	...	..A	...	...	...	...	..G	...	..T	..T	...	..A	...	..A	..G	A.T	..A	...	...	...	...	.CT	
...	...	..C	...	...	...	..A	...	...	...	...	..T	..T	...	..A	..T	..A	T..	A.T	...	C..	..A	...	..A	.CT	
...	..C	...	..C	...	..A	..A	..A	...	...	...	..T	..T	..C	...	..T	...	..G	..T	...	C.T	..A	C..	...	.CT	
...	..C	...	..C	...	..A	..A	..A	...	...	...	..T	..T	..C	...	..T	...	..G	..T	...	C.T	..A	C..	...	.CT	
...	...	...	...	..A	..A	..A	..A	...	...	...	..T	..C	...	...	..T	...	T..	..T	..A	...	..A	C.C	...	.CT	
...	...	...	...	..A	..A	..A	..A	...	...	...	..T	..C	...	...	..T	...	T..	..T	..A	...	..A	C.C	...	.CT	
...	...	...	..C	C.T	..A	..A	..C	...	...	...	..T	..C	...	..G	...	..A	...	...	...	...	..A	C.C	..T	.CA	
...	...	...	..C	C.T	..A	..A	..C	...	...	...	..T	..C	...	..G	...	...	...	...	...	...	..A	C.C	..T	.CA	
..G	...	...	..C	C.T	..A	...	...	...	...	...	..T	..C	...	..A	..T	..A	T..	...	...	...	..A	C.T	..T	.CT	
...	...	...	...	..A	..A	..A	..C	...	...	...	..A	..A	...	..A	..T	..A	T..	..T	...	...	..A	...	..C	.CT	
...	...	...	..C	..A	...	..A	..A	...	...	...	..T	..T	...	..A	...	..A	T..	..T	..G	...	..A	...	...	.CT	
...	...	...	..C	..A	...	..A	..A	...	...	...	..T	..T	...	..A	...	..A	T..	..T	..G	...	..A	...	...	.CT	

(*continued*)

TABLE 2. (*Continued*)

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CCG GAC ATG GCT TTC CCC CGG TTA AAT AAT ATA AGT TTT TGA CTT TTG CCT CCG GCA TTA TGT CTT TTG TTA GCT  285
..A ..T ..A ... ..T ..T ..T ... ... ..C ..G ..A ... ... ..G C.T ... ..T ..G C.T CT. ..A ..A C.T T.C
..T ... ..A ... ... ..T ..A ... ... ... ... ... ..C ... ... ..A ... ..A ..T C.T CTC ..C C.T ... T..
..A ..T ..A ..A ..T ..A ..T ... ... ... ... ..A ... ... T.A C.T ..C ..A ..T C.. .TA T.A C.C C.. T..
..A ..T ..A ..A ..T ..A ..T ... ... ... ... ..A ... ... T.A C.T ..C ..A ..T C.. .TA T.A C.C C.. T..
..A ... ..A ..A ..T ..T ..T ..G ... ... ... ..A ... ... T.A C.T ... ..T ... ... .TG T.G ... ... T..
..T ... ..A ..A ... ... ... C.. ... ... ... ..A ..C ... ... C.T ... ... ... ... .TA T.A C.T C.G T..
..T ... ..A ..A ... ... ... C.. ... ... ... ..A ..C ... ... C.T ... ... ... ... .TA T.A C.T A.G T..
..T ..T ..A ..A ..T ..T ... C.. ... ... ..G ..A ... ... ..C C.T ... ..A ... C.. ..A T.G C.C C.. T.C
..T ..T ..A ..A ..T ..T ... C.. ... ... ..G ..A ... ... ..C C.T ... ..A ... C.. .TA T.G C.C C.. T.C
..A ..T ... ... ..T ..G ..A C.T ... ..C ... ... ..C ... T.A ... ... ..C ..T ... .TA ..G ..A C.. T..
..A ..T ... ... ..T ..G ..A C.T ... ..C ... ... ..C ... T.A ... ... ..C ..T ... .TA ..G ..A C.. T..
..A ..T ..A ... ..T ..T ... C.T ... ..C ... ... ..C ... ... ..A ..C ..T ..T ..G CTA ..A ..A C.T T..
..T ..T ..A ... ..T ..T ..A ... ... ... ... ..A ... ... T.A C.T ... ..T ..T ... .TA T.A ..A ... T..
..A ..T ... ..A ..T ..T ..A ... ... ..C ... ..A ... ... T.A C.T ... ..C ..T ... .TA T.G C.T C.T T..
..A ..T ... ..A ..T ..T ..A ... ... ..C ... ..A ... ... T.A C.T ... ..C ..T ... .TA T.G C.T C.T T..
TCA GGG GCG GTT GAG AGG GGG GCT GGG ACT GGG TGA ACT GTG TAT CCT CCT TTG GCG GGG AAT GTG GGG CAT GCT  360
... .CT ..C ..A ..A ..T ..A .T. ... ..A ..A ... ... ..T ..C ... ..A ... ..T ... ... C.T .C. ... ...
... .CA ..A ..A ... ... ..T .T. ..A ... ..C ... ... ..T ... ... ... ..A ..A ... ... C.A .CT ..C ..A
..G .CA ..A ... ..A ... ..A ..A ..T ..C ..A ... ..A ..C ..C ... ..A C.T ..T ..A ... T.A .CT ... ...
..G .CA ..A ... ..A ... ..A ..A ..T ..C ..A ... ..A ..C ..C ... ..A C.T ..T ..A ... T.A .CT ... ...
... .CT ..T ... ..A ..A ..A ..C ..A ... ..A ... ... ..A ... ..A ..A C.T ..T ..A ... T.A .CT ... ...
..G .CC ..T ... ..A ..T ..T ..A ..T ... ... ..G ..A ..A ..C ..A ..A C.T ..C ..A ... C.A .CC ... ...
..G .CC ..T ... ... ..T ..T ..A ..T ... ... ..G ..A ..A ..C ..A ..A C.T ..T ..A ... C.A .CC ..C ...
... .CT ..C ... ..A ... ..A ..C ..T ..C ..C ... ..A ... ... ..A ..A C.T ..T ..A ... T.A .CT ..C ...
... .CT ..C ... ..A ... ..A ..C ..T ..C ..C ... ..A ... ... ..A ..A C.T ..T ..A ... T.A .CT ..C ...
..T .CA ..A ... ..A ..C ..T ... ..A ..A ... ... ... ... ... ... ... C.T T.T A.C ..C T.. .CA ... ..C
..T .CA ..A ... ..A ..C ..T ... ..A ..A ... ... ... ... ... ... ... C.T T.T A.C ..C T.. .CA ... ..C
..G .CA ..T ..A ... ..A ..A ..A ... ..A ..A ... ..C ... ... ..C ..A ..A T.T A.T ..C A.T .CT ..C ..G
..T .CC ..A ..A ..A ... ..T .TA ..A ..A ..A ..G ... ..A ... ... ... ..A T.T ... ..C T.. .CT ... ...
..T .C. ..C ..A ..A ..A ..A ... ..T ..A ..A ... ... ..A ... ..A ..A ... ..C .CT ... T.. .CC ... ..C
..T .C. .GC ..A ..A ..A ..A ... ..T ..A ..A ... ... ..A ... ..A ..A ... ..C .CT ... T.. .CC ... ..C
GGT GGA TCT GTT GAC TTA GCT ATT TTT TCT TTA CAT TTA GCT GGA GTA TCG TCT ATT TTA GGG GCT GTT AAT TTT  435
..A ..T ..A ..A A.T C.T ..A ... ... ... C.G ... ... ..A ..T ... ..C ... ..C ..G ..T ..A ..A ..C ...
..G ... ..A ..C ... ... ... ... ..C ... C.T ..C C.. ... ..G ..T ..T ... ... ... ... ... ..A ..C ..C
..C ..T ... ... ..T ... ... ... ... ... ... ... ... ... ..T ... ..T ... ... ... ... ... ..A ... ...
..C ..T ... ... ..T ... ... ... ... ... ... ... ... ... ..T ... ..T ... ... ... ... ... ..A ... ...
... ..G ... ... ..T ... ... ... ... ... ... ..C ... ... ..T ... ..T ..A ... ... ..A ... ..A ... ...
... ..C ... ... ..T ... ... ... ... ... ... ... C.G ... ..T ..T ..A ... ... ... ..T ... ..A ..C ...
... ..C ... ... ..T ... ... ... ... ... ... ... C.G ... ..T ..T ..A ... ... ... ..T ... ..A ... ...
... ..T ... ..A ..T ... ... ... ... ... C.. ..C ... ... ..T ..T ..A ... ... ... ..T ... ..A ... ...
... ..T ... ..A ..T ... ... ... ... ... ... ..C ... ... ..T ..T ..A ... ... ... ..T ... ..A ... ...
... ..G ... ..A ..T ... ... ... ... ... C.T ..C ... ... ..T ..T ..T ..A ... C.T ... ... ..A ... ...
... ..G ... ..A ..T ... ... ... ... ... C.T ..C ... ... ..T ..T ..T ..A ... C.T ... ... ..A ... ...
..G ..G ... ..A ..T ... ... ... ... ... C.C ..C ... ..G ..T ..T ..T ... ... C.T ... ... ..A ... ...
..A ..T ... ..A ..T C.T ... ... ... ... ... ... C.. ... ..G ..T ..T ..A ... ... ..A ... ..A ... ...
..A ... ..A ..A ..T ... ..C ... ... ... C.C ... ... ..G ..T ..T ..T ..G ... ... ..T ... ..A ... ...
..A ... ..A ..A ..T ... ..C ... ... ... C.C ... ... ..G ..T ..T ..T ..G ... ... ..T ... ..A ... ...
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ATT ACT ACA ATT GTA AAT ATA CGA AGA GAA GGG ATA CAA TTA GAG CGA TTA CCT TTG TTT GTT TGG TCT GTA AAA  510
... ..A ... ... A.T ..C ... ..T T.G CGT ..T ... ... ..T ... ..G C.T ... ..A ... ..A ... ... ..G ...
... ..C ... ... A.T ..C ..G ... T.. CG. ..A ... ..G ..C ... ..G C.T ... C.T ... ..G ... ..A ... ...
... ..A ..T ... A.T ..C ... ... T.. CGT ..T ... ... ..T ..A ... C.T ..A ..A ... ..A ... ..A ... ...
... ..A ..T ... A.T ..C ... ... T.. CGT ..T ... ... ..T ..A ... C.T ..A ..A ... ..A ... ..A ... ...
... ..A ..T ... A.T ... ... ... T.. CG. ..A ... ... ..T ..A ... C.T ..A ..A ... ... ... ... ... ...
... ... ..T ... A.T ... ... ... T.G CGT ... ..G ... ..T ..A ... C.T ... ..A ... ... ..A ..A ..G .T.
... ... ..T ... A.T ... ... ... T.G CGT ..A ..G ... ..T ..A ... C.T ... ..A ... ... ..A ..A ..G .T.
... ..C ..T ... A.T ... ... ... T.. CGG ..A ... ... ..T ..A ... C.T ... ..A ... ... ... ..G ..G .T.
... ..C ..T ... A.T ... ... ... T.. CGG ..A ... ... ..T ..A ... C.T ... ..A ... ... ... ..G ..G .T.
... ..A ..C ... A.C ... ... ... T.. CG. ..A ... ..G ..T ..A ..G C.T ... ... ... ..C ..A ... ... ..G
... ..A ..C ... A.C ... ... ... T.. CG. ..A ... ..G ..T ..A ..G C.T ... ... ... A.C ..A ... ... ..G
... ..A ..T ..C A.T ... ... ..G T.. CG. ..A ..G ..G ..T ... ..G C.T ..G ... ..C ..A ..A ... ... ...
... ..C ..T ... A.T ... ... ... T.. C.. ..A ..G ... ... ..A ..T A.G ... ..A ... A.. ..A ..G ..T ...
... ... ..T ..C A.T ... ... ... T.. CG. ..T ..G ..G ..T ... ..G C.T ... ..A ... ..A ..A ... ... ...
... ... ..T ..C A.T ... ... ... T.. CG. ..T ..G ..G ..T ... ..G C.T ... ..A ... ..A ..A ... ... ...

ATT ACT GCT ATT TTG TTA TTG CTT TCC TTG CCT GTT TTA GCT GGA GGA ATT ACA ATA TTG TTA ACT GAT CGT AAT  585
... ... ..A ... ..A C.C C.T T.A ..T C.T ... ... ... ... ... .C. ... ... ..G C.A ... ..G ... ..A ..C
..C ... ..C G.. C.T ... C.T ... ... C.. ... ..A C.T ..A ..T .CT ... ..T ..G C.T C.T ... ..C ..G ..C
... ... ..A G.. ..A C.T C.T T.A ... ..A ..G ... ... ... ..G .CT ... ... ... ..A ... ... ... ..A ..C
... ... ..A G.. ..A C.T C.T T.A ... ..A ..G ... ... ... ..G .CT ... ... ... ..A ... ... ... ..A ..C
... ... ..C G.A ... C.T ..A T.A ..T C.T ... ... ... ... ..T .CT ... ... ... C.A ..G ... ... ..A ...
..C ... ... G.. ..A C.C ... ..A ..T C.T ... ... ... ... ..G .CT ... ... ... C.T ... ... ... ..A ...
..C ... ... G.A ..A C.C ... ..A ..T C.T ... ... ... ... ..G .CT ... ... ... C.T ... ... ... ..A ...
... ... ... G.A C.A ... ..A ..A ..T C.T ... ... ... ... ..T .CC ... ... ... C.C C.. ..C ..C ..A ...
... ... ... G.A C.A ... ..A ..A ..T C.T ... ... ... ... ..T .CC ... ... ... C.C C.. ..C ..C ..A ...
... ... ... ... ..A ..G C.A T.A ..T C.A ... ..C ... ..G ... .C. ... ..T ..G C.T C.. ..G ... ..A ...
... ... ... ... ..A ..G C.A T.A ..T C.A ... ..C ... ..G ... .C. ... ..T ..G C.T C.. ..G ... ..A ...
... ... ..C ... C.A ... ... ..A ..T ..A ... ..C ... ... ..T .CT ... ..T ..G C.T ... ..G ... ..A ...
... ... ..A ... ... ... C.C T.A ..T C.T ... ..C C.T ... ..T .C. ... ..T ... C.T ... ..A ... ... ...
... ..A ... ... ..A ... ... ... ..A ..A ..A ..G ... ... ..T .C. ... ..T ..G C.C ... ... ... ..A ...
... ..A ... ... ..A ... ... ... ..A ..A ..A ..G ... ... ..T .C. ... ..T ..G C.C ... ... ... ..A ...

TTT AAT ACT TCT TTT TTT GAT CCT GCN GGA GGG GGG GAT CCT ATT TTA TAC CAA CAT TTG 645
... ... ..A G.A ..C ... ... ..A ..A ..G --- --- --- --- --- --- --- --- --- ---
... ... ..A G.. ..C ..- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
... ... ..A G.. ... ... ... ... ..T ..T ..T ..T ..C ..A ... C.T ..T ... ... C.T
... ... ..A G.. ... ... ... ... ..T ..T ..T ..T ..C ..A ... C.T ..T ... ... C.T
... ... ... G.. ... ... ... ... ..T ..T ..T ..A ... ..A ... C.T ..T ... ... C.T
... ... ..G G.C ... ... ..C ..A ..C ..G ..T ..T ... ... ... ... ... ... ..C C.T
... ... ..G G.C ... ... ..C ..A ..C ..G ..T ..T ... ... ... ... ... ... ..C C.T
... ... ..G G.. ... ... ... ..G ..T ... ..T ..A ... ..A N.. C.T ..T ..G AC. C.T
... ... ..G G.. ... ... ... ..G ..T ... ..T ..A ... ..A .N. C.T ..T ..N AC. C.T
... ..C ... G.. ..C ... ... ... ..A ... ... ... ... ... ... ..G ..T ..G ... C.A
... ..C ... G.. ..C ... ... ... ..A ... ... ... ... ... ... ..G ..T ..G ... C.A
... ... ... G.A ... ..C ..C ..A ..A ..G ..T ..A ..C ... ... ... ... ..G ..C C.T
..A ... ..A G.. ... ... ... ... ..T ..G ..A ..A ... ..A ... ... ... ..G ... C.C
... ... ..G G.. ... ... ..C ... ..T ... ..T ..A ... ... ... C.T ..T ..G ... ..A
... ... ..G G.. ... ... ..C ... ..T ... ..T ..A ... ... ... C.T ..T ..G ... ..A
```

tatively assign this deletion to a compression-based misreading of sequence in a GC-rich region.

Felsenstein's distance matrix of sequence divergence, based on data in Table 2, is given in Table 3. Individuals of a population differed by nucleotide divergence values of 0.005 ± 0.004 (N = 5). The subspecies of *Oncomelania hupensis* differed by 0.148 ± 0.004 (N = 4). The two species of *Hydrobia* differed by 0.162 (range 0.161 - 0.163). The genera *Tricula* vs. *Gammatricula* differed by 0.132 (range of 0.130 - 0.133). The subfamily Pomatiopsinae vs. the Triculinae differed by 0.179 ± 0.020 (N = 12). The families Hydrobiidae vs. Pomatiopsidae differed by 0.267 ± 0.016 (N = 21). The non-rissoacean outgroup snail taxa differed from the rissoaceans by 0.274 ± 0.023.

The unrooted maximum likelihood tree with the polyplacophoran as outgroup is given in Figure 1. Replacement of *Katharina* by *Setia* as the outgroup does not change the topology of the remaining tree. From these trees the following are clear: (1) The pomatiopsid taxa are one distinct clade; the other rissoaceans form a second distinct clade; (2) The truncatellids are more closely allied to the hydrobiids than to the pomatiopsids; (3) The rissoid *Setia* is part of the Truncatellidae-Hydrobiidae clade (except in Fig. 2, where it is the outgroup); (4) The two subspecies of *Oncomelania* are clearly divergent; (5) The triculine taxa appear divergent from the pomatiopsine taxa. However, the triculine node is only weakly supported (54% bootstrapping value).

The Hennig86 analysis using *Setia* as outgroup yielded two equally parsimonious trees with a length of 517, a consistency index of 0.66 and a retention index of 0.74. The Nelson consensus tree is shown in Figure 2. The pomatiopsids are in one clade, the hydrobiids and truncatellids in another. The results are the same as in the maximum likelihood analysis except that the position of *Tricula* is unresolved: there is a trifurcation in the pomatiopsid clade with *Tricula* not unequivocally within a triculine clade (it is in one of the alternative Hennig trees).

The trees obtained using *Stramonita* and *Cerithium* as outgroups are given in Figures 3 and 4. The maximum likelihood analysis (Fig. 3) yields a tree for the ingroup taxa similar to that in Figure 1 except that *Tricula* is a sister taxon to *Oncomelania* rather than to *Gammatricula*. The PAUP-based parsimony analyses (Fig. 4) again clearly define two major clades, a Pomatiopsidae clade and a Truncatellidae-Hydrobiidae clade. The analysis produced two shortest trees of 974 steps; the set of four next shortest trees required one additional step; the latter were not included in computing the Adams consensus tree. The two shortest trees differed in their placement of *Setia* within the hydrobiid clade, thus resulting in a consensus tree with a trifurcation in this clade.

DISCUSSION

Taxonomic decisions should not be made on the basis of molecular distance coefficients alone (Davis, 1994), but on anatomical, cytological and developmental data within an ecological context. Thus, the COI data presented here must be examined in light of the available anatomical data and the patterns of evolution evidenced in the clades shown here. The data provide a beginning of showing relationships; there are insufficient hydrobiid, truncatellid and rissoid genera and species involved in this study to strongly support discrete rissoid, truncatellid, and hydrobiid clades. The data are, however, sufficient to answer our questions and provide the basis for predictions concerning family relationships indicated here.

The hydrobiids and pomatiopsids are distinct clades. The genetic data coupled with anatomical data reviewed in Davis (1979, 1980, 1992) show that these clades are greatly divergent. The COI sequence data strongly support the existence of a pomatiopsid clade separate from the hydrobiids.

The distinctiveness of the pomatiopsid clade is further evidenced by the grouping of the Rissoidae and Truncatellidae with the Hydrobiidae within one clade. Thus, a second question is answered: the Truncatellidae, by these data, are more allied genetically with the Hydrobiidae than with the Pomatiopsidae. However, the truncatellid branch is weakly supported, so additional data are required. Davis (1979) and Ponder (1988) hypothesized that the Truncatellidae were closely related to the Pomatiopsidae (Rosenberg, 1996a). The Pomatiopsidae share with the Truncatellidae and Assimineidae the evolution to terrestriality from aquatic and amphibious habitats in some clades (Rosenberg, 1996b). However, the anatomical data available are insufficient to resolve whether the Truncatellidae are closer phylogenetically to the Hydrobiidae or to the Pomatiopsidae.

TABLE 3. Distance matrix of sequence divergence over 578 nucleotide positions in the cytochrome *c* oxidase I gene under maximum likelihood method.

	Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*K. tunicata*	—															
2	*S. haemastoma*	0.3229	—														
3	*C. atratum*	0.3153	0.2895	—													
4	*G. chinensis* 414/415	0.2597	0.2360	0.2809	—												
5	*G. chinensis*	0.2571	0.2336	0.2784	0.0017	—											
6	*Tricula* sp. 453/454	0.2457	0.2608	0.2800	0.1304	0.1325	—										
7	*O. hupensis hupensis* 93	0.2752	0.2846	0.2838	0.1972	0.1949	0.1902	—									
8	*O. hupensis hupensis* 96	0.2784	0.2957	0.2820	0.2023	0.2000	0.1858	0.0122	—								
9	*O. hupensis robertsoni* 45	0.2698	0.2662	0.2647	0.1745	0.1723	0.1468	0.1526	0.1465	—							
10	*O. hupensis robertsoni* 48	0.2704	0.2661	0.2647	0.1701	0.1679	0.1425	0.1484	0.1424	0.0035	—						
11	*H.* cf. *pontieuxini* 346/347	0.2874	0.3026	0.2936	0.2761	0.2761	0.2353	0.2808	0.2844	0.2567	0.2566	—					
12	*H.* cf. *pontieuxini* 351	0.2850	0.3106	0.2964	0.2788	0.2788	0.2379	0.2835	0.2871	0.2544	0.2343	0.0052	—				
13	*H. neglecta* 435/436/439	0.3098	0.3017	0.2749	0.2665	0.2665	0.2417	0.2834	0.2769	0.2605	0.2605	0.1612	0.1634	—			
14	*S. turriculata* 474/476/477	0.2683	0.2645	0.2771	0.2269	0.2293	0.2051	0.2582	0.2589	0.2431	0.2381	0.2193	0.2218	0.2426	—		
15	*T. pulchella* 479	0.2830	0.2582	0.2872	0.2113	0.2113	0.2014	0.2413	0.2519	0.2237	0.2237	0.2164	0.2235	0.2080	0.2190	—	
16	*T. pulchella* 480	0.2862	0.2613	0.2904	0.2141	0.2141	0.2041	0.2442	0.2549	0.2266	0.2265	0.2192	0.2264	0.2108	0.2218	0.0017	—

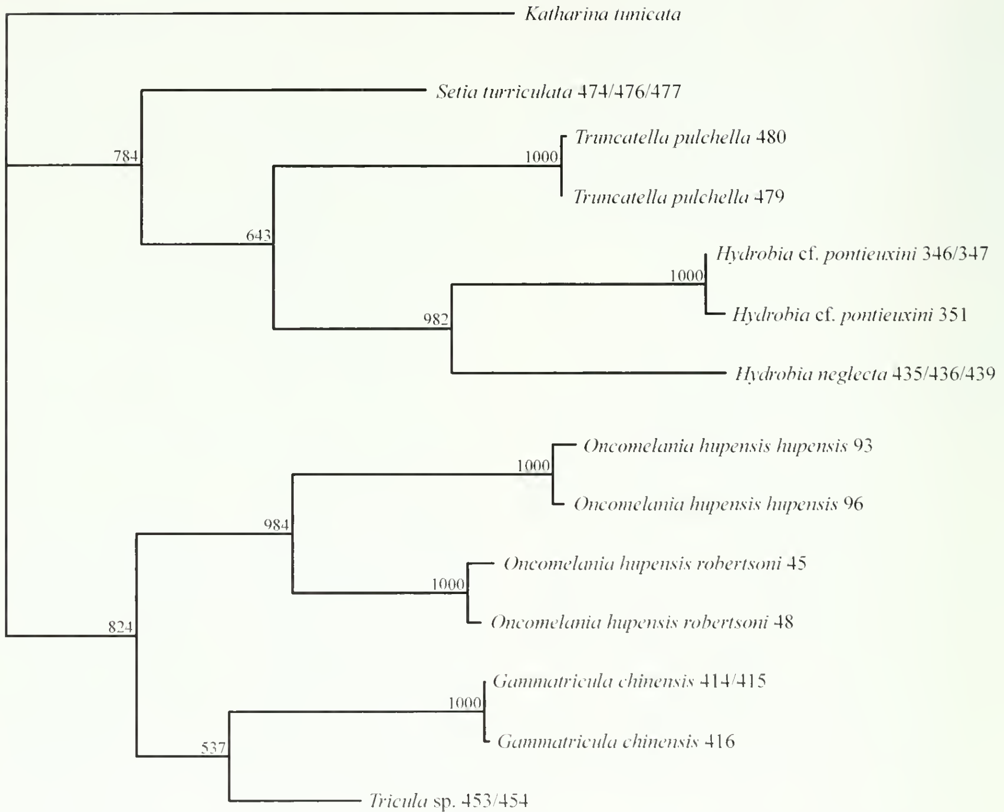

FIG. 1. Unrooted tree based on maximum likelihood with *Katharina tunicata* as the outgroup. Bootstrap values (1,000 replicates) are indicated for each node.

Anatomical data do indicate that the Rissoidae are sister taxa to the Hydrobiidae and the Truncatellidae.

A 28S ribosomal RNA phylogeny placed the Hydrobiidae as a sister group to the Truncatellidae and the Pomatiopsidae, with a terminal *Truncatella*-Pomatiopsidae clade divergent from another *Truncatella* group (Rosenberg et al., 1997). However, as Davis (1994) pointed out, one must be circumspect in assessing phylogenies based on 28S rRNA. In the results presented by both Davis (1994) and Rosenberg et al. (1994, 1997) involving rRNA, there were some considerable surprises not supported by comparative anatomy and allozyme-based phylogenies, due in part to the insufficient quantity of informative characters (85) to resolve a large assemblage (40) of divergent taxa.

In the rRNA data presented by Davis (1994: fig. 10) there were many more differences among margaritiferine taxa than among all other unionid taxa. *Cumberlandia* was basal in the unionid clade, whereas *Margaritifera margaritifera* and *M. falcata* were highly divergent from each other in a margaritiferine clade. The results were as if rRNA evolution went unexplainably berserk (greatly accelerated) in the Margaritiferidae; results not supported by any other data (see also Ledyard et al., 1996). In contrast to the rRNA sequence data, the results with mitochondrial genes have been congruent with results based on other data, and the total weight of evidence leads us to consider the truncatellids within the same clade as, and closely related to the Hydrobiidae, but divergent from the Pomatiopsidae. This is our hypothesis to be challenged with results based on yet other genes.

The COI sequence data are congruent with cytochrome *b* gene sequence data involving populations of *Oncomelania hupensis* from mainland China; the results showed evidence for two distinct subspecies: *O. h. hupensis* and *O. h. robertsoni* (Spolsky et al., 1996). Both data sets are congruent with an al-

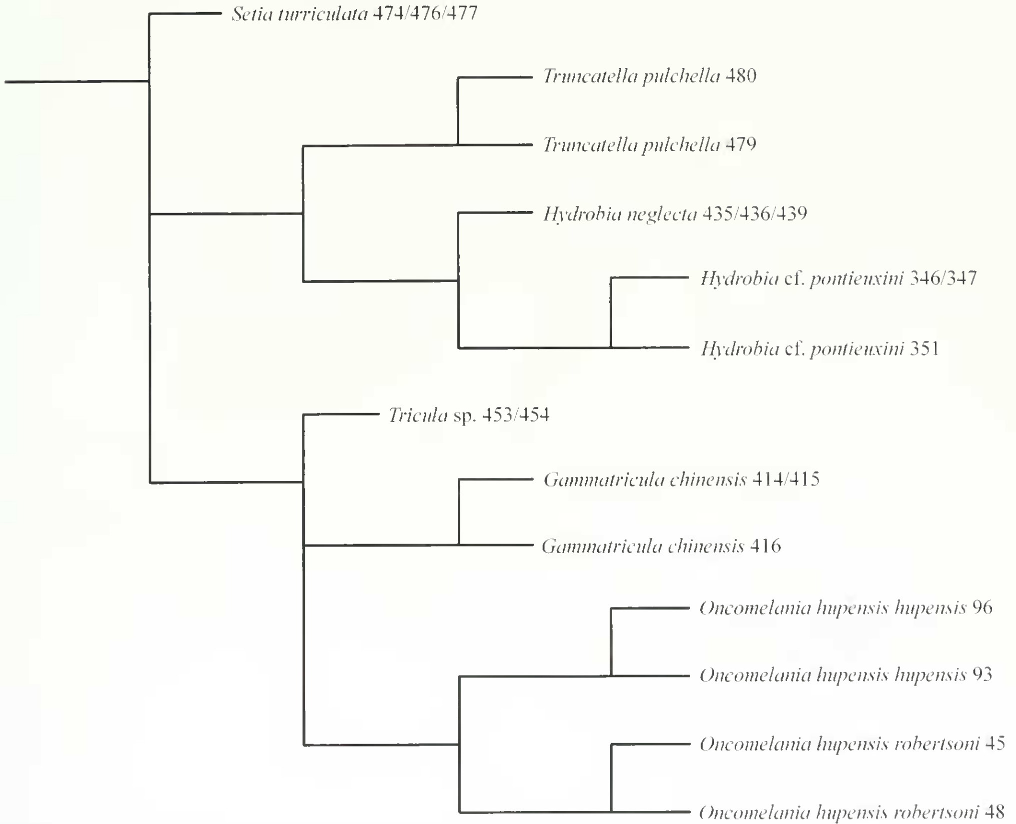

FIG. 2. Unrooted Nelson consensus tree based on Hennig86 analysis, with *Setia turriculata* as outgroup.

lozyme-based phylogeny indicating the occurrence of three subspecies of *Oncomelania hupensis* (the third, *tangi*, from Fujian Province, was not available for the DNA studies). *Oncomelania h. robertsoni* has a smooth shell, no varix, and is relatively shorter in shell length than *O. h. hupensis*, which has strong ribs and strong varix when living in the Yangtze River flood plains, but lacks ribs (although retaining a strong varix) when living above the effects of the annual flooding. *Oncomelania h. robertsoni* lives above the Three Gorges of the Yangtze, whereas *O. h. hupensis* is found below the gorges along the Yangtze River drainages. The distance coefficient between the subspecies using cytochrome *b* data averaged 0.110 (range of 0.1021 - 0.1186), based on *robertsoni* from Yunnan and Sichuan and *hupensis* from Jiang Xi Province. The equivalent average distance in this study is 0.148 ± 0.004 (N = 4) with a range of 0.142 - 0.153. In this study, *robertsoni* came from Yunnan, and *hupensis* from Hubei Province, a province upstream from Jiang Xi. This suggests that the COI gene has diverged more than the cytochrome *b* gene and thus may be more useful to detect population and subspecies divergences and patterns of evolution. That the COI gene appears to have diverged more than the cytochrome *b* is of interest given the conventional understanding that COI tends to be more conservative in most taxa.

The two species of *Hydrobia* differed from each other by a distance coefficient only about 6% greater than the distance between the subspecies of *Oncomelania hupensis*, a surprise given the considerable anatomical differences between the hydrobiid species and the great geographic distance separating them (thousands of ocean miles between Bulgaria and Denmark). In contrast to the large differences between *Oncomelania hupensis* subspecies, two populations of *O. h. robertsoni* separated by over 600 km had a cytochrome *b* difference of only 0.038, a relatively small amount of dif-

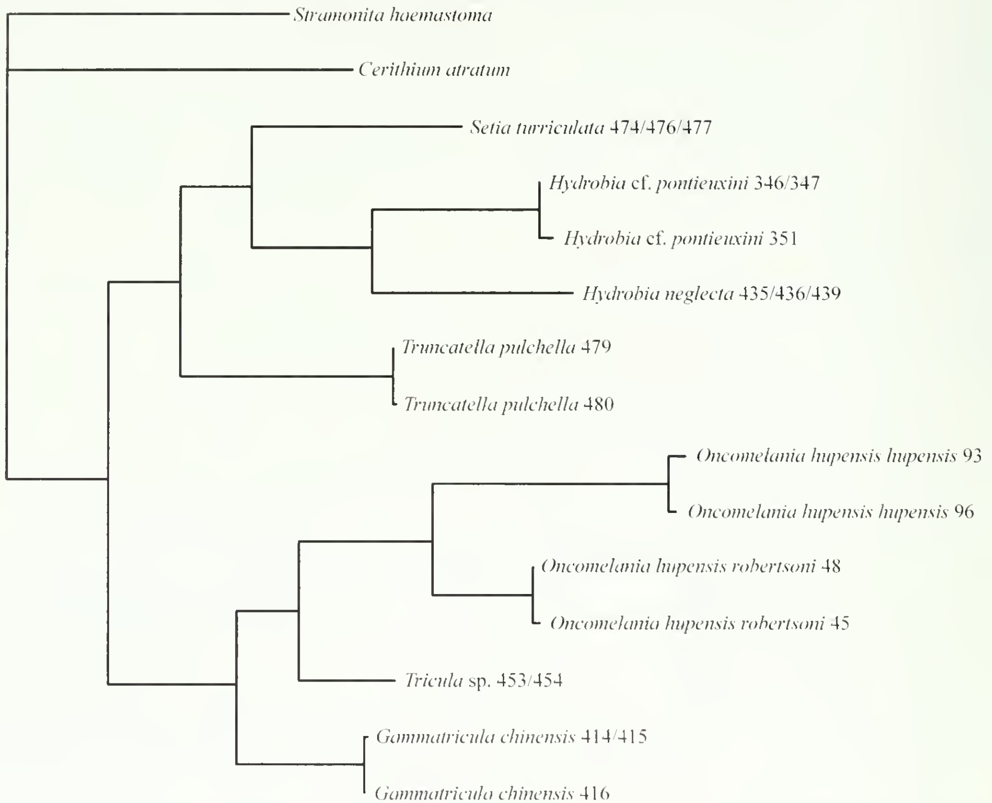

FIG. 3. Unrooted maximum likelihood tree using *Stramonita* and *Cerithium* as outgroups. Character changes were optimally weighted at 1.3:1 for transitions:transversions.

ference. It appears that the subspecies have diverged considerably since *Oncomelania* dispersed into China about 10 million years ago (Davis, 1979, 1980). The considerable genetic divergence is also seen in allozyme data (Woodruff et al., 1988; Davis et al., 1995). The Nei minimum D for *robertsoni* vs. *hupensis* was 0.257 ± 0.077 (N = 21) (Davis et al., 1995). Population variation within *hupensis* was 0.160 ± 0.085 (N = 21). Davis et al. (1995) discuss why we do not consider these taxa as full species. Our question here is what has driven such large genetic divergence when the snails are identical morphologically and anatomically (except for size and presence of ribs on some populations) and can replace each other ecologically? One hypothesis is that, as virtually all populations of *Oncomelania hupensis* throughout its range have been heavily infected with *Schistosoma japonicum,* coevolutionary pressures have been the cause. Added to this are the natural geographical isolation of Yunnan and Sichuan from the central Yangtze River basin and the mountain ranges that separate Fujian Province from the other two regions. We now need considerable population studies of COI sequences to assess the extent of population divergence across China.

Considering the Triculinae issue, it could be argued that, on the basis of anatomy, the Triculinae and Pomatiopsinae are not historically closely related. The pomatiopsine female reproductive system appears considerably different from that of the Triculinae, and what Davis has called the spermathecal duct in each taxon may not be homologous. The data presented here are congruent with allozyme data of Davis et al. (1994) that show general agreement of phylogenies based on both anatomical and allozyme data: *Oncomelania hupensis* is in a clade apart from the three triculine taxa in the allozyme study, *Gammatricula chinensis, Gammatricula songi,* and *Neotricula lilii.* However, the two clades are very close genetically. The Nei D between *On-*

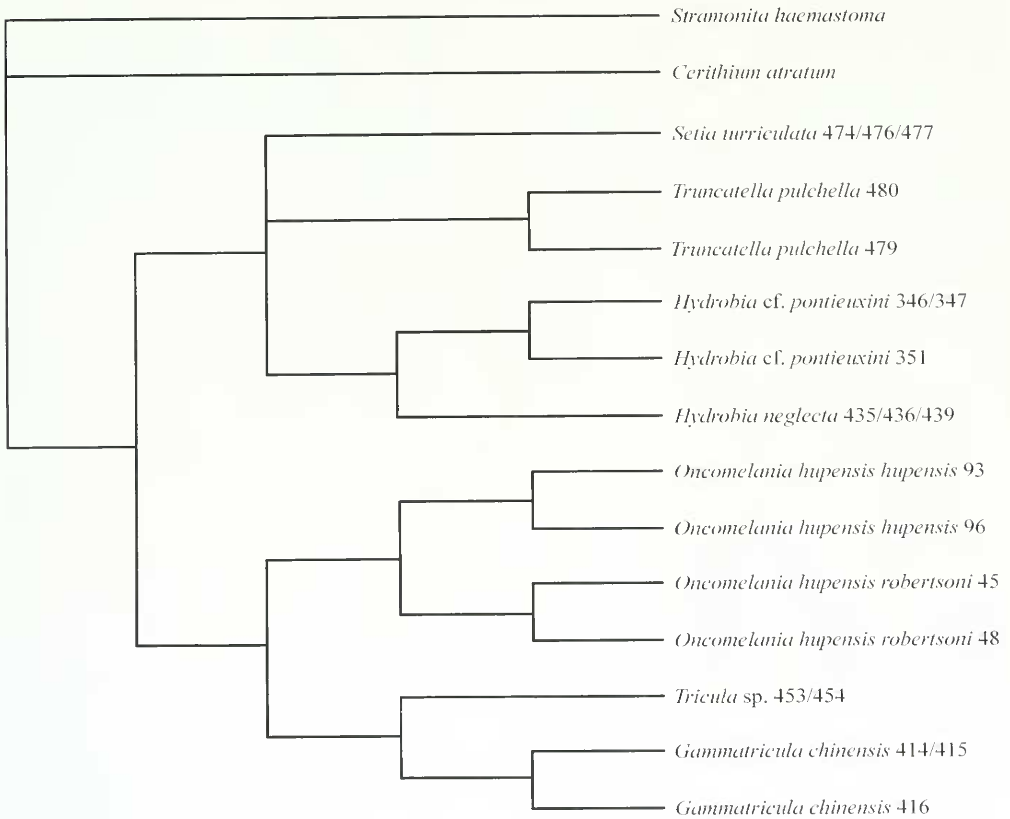

FIG. 4. Unrooted PAUP tree: Adams consensus of two shortest trees produced by a branch and bound search, with transitions and transversions weighted equally.

comelania and the three triculines was 1.293 ± 0.412 (1.000 - 1.764); D among triculines was 0.890 ± 0.301 (0.689 - 1.236). Further, *Oncomelania* was less distant from *Gammatricula chinensis* (1.116) than was *Neotricula lilii* (1.236). Accordingly, the phenogram based on Nei's D does not provide a great separation from the triculine taxa because of the closer relationship of *Oncomelania* to *N. lilii*.

The *Tricula* of this study is a new species that will be described elsewhere; anatomical data confirm its placement in *Tricula*. Hennig86 produced two trees because *O. h. robertsoni* was closer to *Tricula* sp. (0.144) than it was to *O. h. hupensis* (0.150). The two triculine taxa differed by only 0.132. This very close relationship between *Oncomelania* and some triculine taxa shows the overall close relationship between the two pomatiopsid subfamilies and possibly points the way to unraveling the pathway of evolution of the triculines from early pomatiopsids. For example, the Sichuan *Tricula* is, on the basis of anatomy, closely related to Yunnan species of *Tricula* and to the type species, *Tricula montana* from northern India. The average COI distance of *Tricula* sp. from *O. h. hupensis* is 0.188, whereas that of *Gammatricula chinensis* is 0.195; the same set of relationships to *O. h. robertsoni* is 0.144 and 0.171. The premise that emerges from these data is that *O. h. robertsoni* in Yunnan, with its generalized morphological features, is closer genetically to the basal *Oncomelania* stock that gave rise to *Tricula* than is the derived *Gammatricula* and derived *O. h. hupensis* below the Three Gorges. This premise and initial data are consistent with the hypotheses of Davis (1979, 1980, 1992) that pomatiopsids were introduced into Asia from the northeastern Indian Plate with the Himalayan orogeny, with subsequent evolution and dispersal down river systems. The closer genetic relationships of some triculine taxa to *Oncomelania* points the way to assessing the coevolution of *Schisto-*

soma spp. with both *Oncomelania* and Triculinae.

All the phylogenetic analyses combined affirm the monophyly of the Pomatiopsidae, although the placement of *Tricula* within this clade remains unresolved: using *Katharina* or *Setia* as outgroups in a maximum likelihood analysis, *Tricula* clusters with *Gammatricula;* using the closer *Stramonita* and *Cerithium* as outgroups, *Tricula* clusters with *Oncomelania* in a maximum likelihood analysis, but with *Gammatricula* in a parsimony analysis; a Nelson consensus tree of Hennig86 analyses leaves relationships within the Pomatiopsidae as an unresolved trifurcation. In the end, the bootstrap value for the *Tricula* clade tells the story; placement of *Tricula* depends on the method and choice of outgroups. It is not well resolved within the pomatiopsid clade, but all evidence places it in this clade. This question may best be answered by increasing the number of populations and species of *Tricula* and *Gammatricula* in the analyses, increasing the number of nucleotides analyzed, and sequencing an independent nuclear gene.

All five analyses place *Truncatella* in a clade with *Hydrobia*. The placement of *Setia*, however, is problematic: in analyses where it is not used as an outgroup, it clusters with the hydrobiid-truncatellid clade, but its relationships within this clade are uncertain. The unresolved trifurcations are consistent with weak bootstrapping support for those nodes in the maximum likelihood analyses.

Because the distances of *Katharina* to other taxa are not much different than distances among some ingroup members, we examined the number and kind of substitutions occurring in the COI gene. Substitution rates varied tremendously among codon positions, with strong constraints on amino acid changes: 203 of the 215 third codon positions were variable, while 51 of 215 first codon positions and only ten second codon positions were variable. Translation of the nucleotide sequences clearly pointed out the strong constraints on amino acid changes in this gene: the majority of changes were synonymous substitutions. Among this group of 16 taxa, 40 amino acid positions were variable, of which too few (28) were phylogenetically informative. PAUP analysis of the amino acid sequences produces 28 equally parsimonious shortest trees, resulting in a consensus tree with no resolution.

In an attempt to avoid saturation effects on phylogenetic tree construction, we carried out maximum likelihood analyses using only first and second codon positions: again, these analyses resulted in non-robust and implausible trees with poorly supported nodes, although in this case, the pomatiopsid clade does hold together. We plotted pairwise transition/transversion (Ts/Tv) ratios vs. pairwise distances to look for evidence of substitutional saturation. Although saturation must be occurring only at third codon positions, pairwise ratios were based on the whole sequence, as that is what the phylogenetic analyses were based on. Saturation appears to be occurring in pairwise comparisons with the three outgroup taxa *Katharina, Cerithium,* and *Stramonita,* with a mean Ts/Tv ratio of approximately 1.1, and pairwise differences greater than about 130 nucleotides. For ingroup comparisons, pairwise differences were usually less, varying from about 140 to 1. Ts/Tv ratios were roughly correlated with nucleotide differences, such that ratios approached saturation at distances greater than approximately 120 nucleotide differences. Thus, there appears to be a quantitative difference between ingroup and outgroup taxa in the substitution level. The existence of a discrete ingroup provides further support for the robustness of the phylogeny of this group. Further, although the effect of saturation is to shorten branch length, in this case it is not likely to have affected the topology, that is, branching pattern, of the trees because most of the deep branches are sufficiently long to retain the correct branching pattern.

This study should conclusively clarify two points: (1) The Hydrobiidae are not closely related historically, biogeographically, or genetically to the Pomatiopsidae; they are distinctly different families. (2) Schistosomes have evolved with the pomatiopsid lineage in Asia; hydrobiids do not transmit schistosomes. As implied by the term coevolved, there is a historical genetic linkage between the pomatiopsids and schistosomes extending back to Gondwanaland. With the progression of time, this linkage has deepened; once lost, it cannot be restored (Davis, 1992). Thus, we predict that no hydrobiid suddenly *de novo,* can become a host for any schistosome.

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